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Fatty Acid Alterations and Polymyxin B Binding by Lipopolysaccharides from *Pseudomonas aeruginosa* Adapted to Polymyxin B Resistance

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Lipopolysaccharides were extracted from freeze-dried cells of *Pseudomonas aeruginosa* PAO1 (polymyxin B susceptible), isolate A (polymyxin B resistant), and isolate A-reverted (polymyxin B intermediate resistance) by either the phenol-chloroform-petroleum ether or the modified phenol-water method. Isolate A and isolate A-reverted had drastic losses of 2-hydroxydodecanoic acid and significant decreases in 3-hydroxydecanoic acid. Concentrations of amide-linked 3-hydroxydecanoic acid were similar in all three strains. Minor alterations were noted in the composition of 3-deoxy-D-manno-2-octulosonic acid, heptose, phosphate, neutral sugars, and amino compounds. The concentrations of rhamnose in isolate A and of rhamnose and glucose in isolate A-reverted were significantly different from those in PAO1. Trace amounts of mannose and other minor unidentified carbohydrates were detected in all strains. Polymyxin B included in isolate A growth medium complexed with lipopolysaccharides and remained bound throughout purification. PAO1 lipopolysaccharides bound more polymyxin B than did isolate A lipopolysaccharides. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated minor differences in smooth- and rough-form lipopolysaccharides of the different strains. We propose that loss of hydroxy fatty acids from lipopolysaccharides perturbs outer membrane hydrophobicity and is a contributing factor to polymyxin B adaptive resistance.

There are at least two mechanisms by which *Pseudomonas aeruginosa* acquires resistance to polymyxin B (PB) (17). In the first process, *P. aeruginosa* may develop PB resistance by mutation. Mutational PB resistance can be delineated from the adaptive form of resistance by its inheritability, lower levels of resistance, and lack of distinctive lipid alterations. In the second process, PB-susceptible strains may be stepwise adapted to increased levels of resistance. This form of resistance is uninheritable, and high levels of resistance are maintained only as long as PB is included in the growth media. Similar examples of adaptive resistance to polymyxins or aminoglycosides or both have been reported in *P. aeruginosa* (1), *Escherichia coli* (10), and *Proteus* strains (28).

Adaptive resistance to PB in *P. aeruginosa* is a poorly understood phenomenon characterized by perturbations in ultrastructural architecture (6, 9), outer membrane proteins (8), divalent cations (4), readily extractable lipids (3), and lipopolysaccharides (LPS) (8). These anatomical and physiological considerations plus the absence of inactivating enzymes (7) leave little doubt that PB resistance in *P. aeruginosa* is a function of exclusion by the outer membrane to protect the otherwise sensitive cytoplasmic membrane. Permeation of PB through the outer membrane could, theoretically, be facilitated by any of three generally accepted pathways (18), which include the hydrophobic, hydrophilic, and self-promoted pathways. The amphiphilic nature of PB with its polycationic head group and hydrophobic acyl side chain eliminates the hydrophilic pathway from further consideration (23, 29, 30). The high level of intrinsic resistance of *P. aeruginosa* to hydrophobic antibiotics such as erythromycin and fusic acid indicates that the normal hydrophobic pathway is of minor consequence in wild-type strains (20).

The self-promoted uptake pathway as proposed by Moore et al. (18) hypothesizes that permeabilizers such as PB compete for divalent cation-binding sites within the outer membrane. The displacement of these cations would destabilize the cross bridging between adjacent LPS molecules, allowing the passage of the permeabilizing agent. If this is the case, elimination or modification of potential PB-binding sites on LPS would obviously influence susceptibility. Conrad and Gilleland (4) determined that potential effectors such as divalent cations and acidic phospholipids were drastically reduced in cells adapted to PB resistance and rebounded to near wild-type levels in reverted cells grown in the absence of antibiotic. The interaction of these compounds with LPS implies a significant role for LPS in PB resistance.

The purpose of this study was a comparative analysis of LPS extracted from *P. aeruginosa* strains which were susceptible to PB, adapted to a high level of resistance, or reverted to an intermediate level of susceptibility. These analyses included fatty acid profiles, chemical composition, PB-binding capacity, and quantitation of PB complexing with LPS during growth.

MATERIALS AND METHODS

Strains. The strains of *P. aeruginosa* used in this study have been previously described by Gilleland and Farley (7). The PB-resistant isolate A was derived from the PB-susceptible PAO1 strain by stepwise adaptation to increased amounts of PB. The lack of inheritability indicates that isolate A is not a stable genetic mutant but rather represents an adaptive response to the presence of 6,000 U of PB per ml of growth medium. Isolate A-reverted was derived from isolate A cultured in antibiotic-free medium. Isolate A-

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reverted has an intermediate level of PB susceptibility. The MICs for these strains were determined by Gilleland and Farley (7) for a number of antimicrobial agents by serial twofold dilution using the method of Bailey and Scott (2). The MICs of PB for PAO1, isolate A, and isolate A-reverted were, respectively, 6, 5,000, and 250 μg/ml (1 μg is ca. 8 U). Isolate A became more susceptible to 8 of the 10 hydrophilic antibiotics tested.

Growth medium. All *P. aeruginosa* strains were grown in a glucose-salts basal medium previously described as "basal medium 2" by Gilleland and Lyle (8). Isolate A was grown in basal medium plus 6,000 U of PB per ml added separately after filter sterilization. PB sulfate was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Batch cultures of cells were routinely grown with vigorous aeration at 33 to 35°C in 4-liter Erlenmeyer flasks containing 2 liters of medium. Cells were harvested after they reached the mid- to late-log phase, washed once with distilled water, and freeze-dried.

LPS extraction. Freeze-dried cells of *P. aeruginosa* PAO1, isolate A, and isolate A-reverted were extracted sequentially by the phenol-chloroform-petroleum ether method of Galanos et al. (5), the phenol-water procedure of Westphal and Jann (36), and by a modified phenol-water procedure described as follows. The freeze-dried L_1 to L_4 supernatant fractions were combined and then solubilized (1%, wt/vol) by 90% phenol. LPS was precipitated by the dropwise addition of water, washed twice with 80% phenol, filtered, washed with acetone, and dried in vacuo. An aqueous solution of the filtrate was adjusted to pH 6.5 with 1 N NaOH, boiled for 3 min, and immediately cooled in an ice bath. Preparative centrifugation and ultracentrifugation proceeded by standard procedures. LPS used in subsequent chemical analyses of PAO1 and isolate A-reverted was extracted by the modified phenol-water procedure. LPS used in the analyses of isolate A was extracted by the phenol-chloroform-petroleum ether method. Sample selection for chemical analyses was determined by yield and purity as measured by protein and nucleic acid contamination.

Analytical methods. Fatty acids were analyzed by gas-liquid chromatography by the methods of Rietschel et al. (24) and Wollenweber and Rietschel (Methods Carbohydr. Chem., in press). Total fatty acids were released from LPS by transesterification in methanolic HCl at 85°C for 10 h. Ester-bound fatty acids were cleaved and methylated by 0.5 M $NaOCH_3$ (16 h, 37°C, mild agitation). The resultant sediment representing de-*o*-acylated LPS (LPS-OH) was washed successively three times with cold methanol and three times with cold acetone and freeze-dried in aqueous solution. Amide-bound fatty acids were liberated from LPS-OH by the same acid methanolysis protocol used to analyze total fatty acids. Complete conversion to the methyl ester was ensured in all preparations by the addition of the diazomethane reagent (24). Heptadecanoic and 2-hydroxytetradecanoic acids were used as internal standards for all preparations. The content of 3-deoxy-D-manno-2-octulosonic acid (KDO) was determined by the method of Karkharis et al. (11) with the ammonium salt of KDO (Sigma) as a standard. Phosphorus was quantitated by the technique of Lowry and Tinsley (15). Neutral sugars were analyzed by gas-liquid chromatography by the protocol of Strittmatter et al. (31). The alditol acetate derivatives were prepared for gas-liquid chromatography by the method of Sawardeker et al. (27). Xylose was added as an internal standard. Hexosamine was determined by the Morgan-Elson reaction as described by Strittmatter et al. (31). Unknown carbohydrates and fatty acids were tentatively identified by comparison with authentic standards and cochromatography of samples and standards. Qualitative and quantitative amino compound analyses of LPS samples were performed after acid hydrolysis (4 N HCl, 100°C, 18 h) with a Kontron (Anacomp) automatic amino acid analyzer. Protein was determined by the automatic amino acid analyzer.

Analysis of PB. Samples known or suspected to contain PB were digested and analyzed by the amino acid analyzer in the same manner as described above for other amino compounds. PB was quantitated by a standard curve in which 5.92 residues of diaminobutyrate equal one molecule of PB. The curve was linear between 10 and 1,200 μg of antibiotic. The proportionality between diaminobutyrate and the other primary amino acids composing the PB peptide (leucine, threonine, phenylalanine) was stoichiometric between these same concentrations.

Detection of PB bound to LPS during growth. Amino analysis of isolate A LPS indicated the presence of PB, which had complexed with the LPS during growth. This was confirmed by gently stirring 2 mg of isolate A LPS in 2 ml of 1 N HCl for 20 min at 4°C. Particulate material and supernatant were separated by centrifugation and dried in vacuo over KOH. Precipitate and supernatant were hydrolyzed in 1 ml of 4 N HCl for 18 h at 100°C and analyzed by an automatic amino acid analyzer.

Determination of PB-binding capacity of LPS. The PB-binding capacities of PAO1 LPS and isolate A LPS were determined by adding 4 mg of LPS to 20 mg of PB in 10 ml of aqueous solution. LPS and PB solutions were both sonicated before mixing to achieve an even suspension. Precipitation proceeded for 2 h at ambient temperature with occasional shaking. The precipitate was washed three times with 10 ml of cold water and freeze-dried. Digestion and analysis proceeded in the previously described manner.

SDS-PAGE analysis of LPS. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of LPS were done in a discontinuous buffer system with 5% stacking and 14% separating gels by the methods of Laemmli (14). Each well was loaded with 1 to 5 μg of LPS. The current was 15 mA for the stacking gel and 20 mA for the separating gel. LPS bands were visualized by silver stain as described by Tsai and Frasch (32).

RESULTS

Fatty acid analysis. Biochemical analyses of LPS fatty acids (all comparisons relative to strain PAO1) indicated that isolate A LPS and isolate A-reverted LPS suffered an almost complete loss of 2-hydroxydodecanoic acid, as well as significant decreases in total 3-hydroxydecanoic acid (Table 1). A significant decrease was noted in the concentration of dodecanoic acid in isolate A-reverted LPS. These specific alterations were reflected in an overall quantitative decrease in the LPS fatty acids of isolate A and isolate A-reverted (Table 2). The concentration of the amide-linked 3-hydroxydodecanoic acid was similar in all three strains. Selective hydrolysis of LPS indicated that all other fatty acids were bound by ester linkages.

Chemical composition of LPS. The concentrations of rhamnose in isolate A and glucose and rhamnose in isolate A-reverted were significantly different from those in PAO1 (Table 2). Trace amounts of mannose and other minor unidentified carbohydrates were detected in all strains. LPS amino compounds including glucosamine, galactosamine,

TABLE 1. Fatty acid composition of LPS from *P. aeruginosa* strains with various susceptibilities to PB

Strain	Analytical procedure	Fatty acid (nmol/mg of LPS)[a]				
		$C_{12:0}$	$C_{16:0}$	3-OH $C_{10:0}$	2-OH $C_{12:0}$	3-OH $C_{12:0}$
PAO1	Total	177.1 ± 15.0	12.4 ± 7.0	269.0 ± 11.7	200.7 ± 7.1	345.6 ± 16.1
	Ester bound	112.9 ± 24.8	5.4 ± 3.7	159.5 ± 36.1	129.1 ± 28.5	2.4 ± 0.7
	Amide bound	10.1 ± 3.6	13.8 ± 7.0	12.1 ± 1.4	6.5 ± 0.4	230.9 ± 31.9
Isolate A	Total	198.8 ± 35.5	33.1 ± 9.3[b]	155.5 ± 26.2[b]	5.2 ± 1.9[b]	320.1 ± 69.7
	Ester bound	152.5 ± 23.4	16.5 ± 3.1	154.2 ± 21.9	2.2 ± 0.4	8.2 ± 2.8
	Amide bound	12.9 ± 6.9	10.2 ± 1.9	8.6 ± 2.1	3.5 ± 1.5	248.7 ± 36.1
Isolate A-reverted	Total	75.4 ± 22.5[b]	18.5 ± 9.8	164.1 ± 21.7[b]	4.3 ± 0.6[b]	270.5 ± 26.0
	Ester bound	66.5 ± 15.0	12.9 ± 3.2	159.7 ± 37.3	3.3 ± 1.1	4.0 ± 1.4
	Amide bound	13.3 ± 11.2	6.3 ± 2.2	17.1 ± 2.3	3.2 ± 0.7	225.9 ± 22.4

[a] Means ± standard deviation from three to six independent determinations of each analytical procedure.
[b] Significantly different ($P < 0.05$) from total fatty acid of PAO1 wild type as determined by Duncan's multiple-range test.

and alanine were analyzed, with only minor differences noted among the different strains. The levels of KDO, heptose, and phosphate were also similar in all strains.

PB-LPS complexes in cells grown in presence of PB. LPS extracted from isolate A was first thought to contain significant quantities (7.9%) of protein (Table 2). Subsequent investigations determined that over 90% of this apparent protein contamination was PB which had complexed with LPS during growth (Table 3). The affinity of the PB-LPS complex was demonstrated by its resistance to the rigors of extraction and persistence of PB even after acid treatment. The latter procedure had little effect on the relative ratio of the other amino compounds analyzed.

PB binding to LPS of susceptible and resistant strains. LPS extracted from PB-susceptible PAO1 cells bound approximately 22% more PB than did LPS from PB-resistant isolate A cells (Table 4). These same data indicated that the number of PB-binding sites varied from 3.7 sites per molecule of PAO1 LPS to 2.9 sites per molecule of isolate A LPS. These results must be interpreted cautiously since isolate A was grown in the presence of PB (6,000 U/ml of growth medium) and the resultant extracted LPS was complexed with significant amounts of PB (ca. 75 nm of PB per mg of LPS). Binding of PB has been previously reported to rigidify LPS by altering the overall charge and perturbing the membrane packing arrangement (21). It was not determined whether the reduced PB binding demonstrated by isolate A LPS resulted from architectural changes induced by PB-LPS complexes formed during growth or was due to an actual reduction in the number of available binding sites.

LPS extraction. LPS was extracted from cells of isolate A by the phenol-chloroform-petroleum ether method (1.4% yield). The LPS was a white-gray granular material. LPS was successfully extracted by 90% phenol from the combined L_1 to L_4 supernatant fractions of PAO1 and isolate A-reverted cells. Extraction of PAO1 and isolate A-reverted cells gave respective 2.0 and 0.4% yields of typical fluffy white material.

SDS-PAGE analysis. SDS-PAGE analysis indicated that PAO1 had more smooth-form LPS than did isolate A but less than isolate A-reverted (Fig. 1). LPS from PAO1, isolate A, and isolate A-reverted exhibited various degrees of heterogeneity with respect to smooth-rough forms. This heterogeneity may have been influenced by the different methods used to extract LPS. Isolate A was extracted by the phenol-chloroform-petroleum ether method, which would be expected to select rougher LPS.

TABLE 2. Chemical composition of LPS from *P. aeruginosa* strains with various susceptibilities to PB

Component	Composition (% by wt)[a]		
	PAO1	Isolate A	Isolate A-reverted
Glucose	8.1 ± 0.4	7.3 ± 0.4	7.1 ± 0.4[b]
Rhamnose	5.4 ± 0.2	8.2 ± 0.5[b]	7.5 ± 0.8[b]
Heptose	2.4 ± 0.5	2.7 ± 0.8	3.2 ± 1.1[b]
Mannose	tr	tr	tr
Glucosamine	4.5 ± 0.5	4.8 ± 0.3	4.0 ± 0.8
Glucosamine-phosphate	1.4 ± 0.3	2.1 ± 0.2[b]	1.3 ± 0.0
Alanine	1.5 ± 0.4	1.4 ± 0.1	1.2 ± 0.2
Galactosamine	2.4 ± 0.2	2.5 ± 0.2	1.9 ± 0.6
Hexosamine	7.9 ± 0.5	7.6 ± 0.5	8.0 ± 0.1
KDO	4.3 ± 0.2	4.4 ± 0.2	4.0 ± 0.4
Phosphate	13.1 ± 2.1	13.0 ± 0.5	13.4 ± 0.9
Protein	1.3 ± 1.2	7.9 ± 1.4	2.1 ± 0.9
Fatty acids[c]	22.1	15.8	11.8

[a] Means ± standard deviation from three to five independent determinations of each component.
[b] Significantly different ($P < 0.05$) from value of component in PAO1 wild type as determined by Duncan's multiple-range test.
[c] Summation of total fatty acids (Table 1).

DISCUSSION

LPS extracted from a *P. aeruginosa* strain adapted to PB resistance (isolate A) had a near-total loss of 2-hydroxydodecanoic acid and partial loss of 3-hydroxydecanoic acid (Table 1). Removal of PB from the growth medium (isolate A-reverted) did not restore levels of LPS fatty acids to that observed in the parental strain. This unresponsive reaction

TABLE 3. Persistence of PB in LPS extracted from *P. aeruginosa* isolate A grown in presence of PB

Fraction	Component (nmol/mg of LPS)[a]			
	PB	Glucosamine[b]	Alanine	Galactosamine
Complete LPS	75.5 (0.54)	333.3 (2.38)	153.4 (1.10)	140.0 (1.00)
Precipitate after acid treatment	22.8 (0.27)	186.9 (2.25)	105.8 (1.28)	82.9 (1.00)
Supernatant	43.8 (4.99)	14.6 (1.66)	3.4 (0.38)	8.8 (1.00)

[a] Molar ratio in parentheses relative to galactosamine.
[b] Includes both glucosamine and glucosamine-phosphate.

TABLE 4. PB-binding capacity of LPS from *P. aeruginosa* PAO1 and isolate A

Strain	Composition (nmol/mg of LPS)			Molar ratio (PB/LPS)[a]
	PB	Phosphate	KDO	
PAO1 (PB susceptible)	413.8	1,347.4	170.6	3.7
Isolate A (PB resistant)	317.1[b]	1,315.8	182.8	2.9

[a] Calculated by assuming, as proposed by Peterson et al. (21), 12 residues of phosphate per molecule of LPS. LPS molecular masses for PAO1 and isolate A were, respectively, 8,900 and 9,100 daltons.

[b] Includes PB (ca. 75 nmol/mg of LPS) persisting in LPS extracted from cells grown in presence of PB.

was in marked contrast to the antibiotic-induced alteration of other outer membrane components (phospholipids, divalent cations), which rebounded to near wild-type levels in antibiotic-free media (4). The fatty acid profile of *P. aeruginosa* PAO1 was quantitatively consistent with previous analyses (12). Total 3-hydroxydecanoic acid was significantly higher in PAO1 (Table 1) than in the resistant strains even though the levels of the corresponding ester and amide-bound fatty acid were similar in all strains. Current models of *P. aeruginosa* lipid A (22) indicate that the absence of 2-hydroxydodecanoic acid and reduction of 3-hydroxydecanoic acid would result in a loss of 3-acyloxyacyl residues from PB-resistant (isolate A) and PB intermediate-resistant (isolate A-reverted) strains, which may contribute to this incongruity. The loss of 2-hydroxydodecanoic acid from resistant strains was of interest since increased levels of this fatty acid have been associated with supersusceptibility in *P. aeruginosa* Z61 (13).

The binding of two amphiphilic molecules, such as PB and LPS, involves interaction between the respective hydrophobic and hydrophilic regions of each. The contribution of each region to this complex has been explored by Vaara and colleagues (33–35), who investigated structural and permeability changes associated with PB by using the PB nonapeptide (PB derivative lacking acyl side chain). The results implicated the hydrophobic side chain as a vital component in the binding and ultimate expression of biological activity of PB in the outer membrane. There are strong indications that the acyl side chains of LPS make an equally important contribution to this hydrophobic interaction. Morrison and Jacobs (19) reported a significant decrease in the PB-binding capacity of LPS after alkaline hydrolysis of ester-linked fatty acids. These results strongly suggest that the complete expression of biological activities of LPS-PB complexes depends on a hydrophobic microdomain formed by the interaction of lipophilic side chains found on both. The importance of these hydrophobic interactions is emphasized by the similar levels of KDO and phosphate in all strains analyzed (Table 2). The concept that PB resistance is the sole responsibility of an ionic barrier formed by KDO and phosphate residues is inconsistent with the chemical similarities of these strains, which have tremendous variances in susceptibility. The loss of 3-acyloxyacyl residues would reduce the overall hydrophobicity of resistant LPS and possibly contribute to the lower PB-binding capacity of isolate A. The numbers of PB-binding sites per molecule of LPS from PB-susceptible (3.7) and PB-resistant (2.9) *P. aeruginosa* strains were still considerably higher than the 1 to 2 binding sites reported on the LPS of various enterics (19, 25). The number of PB-binding sites reported here is comparable with the approximately four PB-binding sites reported for *P. aeruginosa* by Moore et al. (16) using another method.

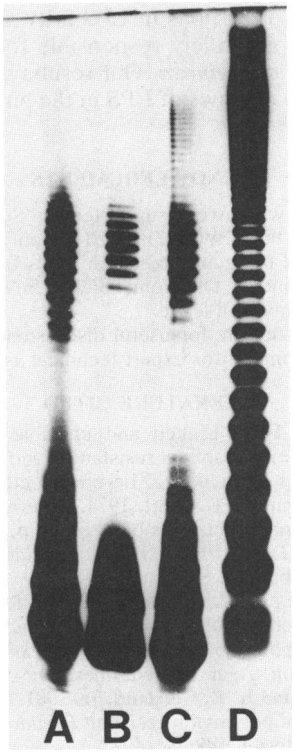

FIG. 1. LPS from *Salmonella abortus equi* control strain and *P. aeruginosa* strains with various susceptibilities to PB analyzed by SDS-PAGE and periodate-silver staining. Lanes: A, PAO1 (PB susceptible); B, isolate A (PB resistant); C, isolate A-reverted (intermediate PB resistance); D, *S. abortus equi* control (smooth form). Lanes A to C (*P. aeruginosa* strains) were loaded with 5 μg of LPS, and lane D (*S. abortus equi*) was loaded with 1 μg of LPS. This concentration of LPS was necessary to visualize the LPS from the smooth populations of *P. aeruginosa* strains but resulted in a slight overloading of the rough populations. Note that resistant strain (isolate A) LPS has a small but reproducible decrease in the smooth form in comparison with both the parent (PAO1) and revertant (isolate A-reverted) strains.

The chemical analyses of all strains were in general agreement with previous analyses of *P. aeruginosa* (12). If rhamnose is assumed to be a major constituent of the O side chain, the concentrations of rhamnose in isolate A and isolate A-reverted were somewhat higher than would be expected from the smooth-rough profiles depicted by SDS-PAGE LPS analysis (Fig. 1). This characterization is, however, consistent with that of Yokota et al. (37), who described a rhamnose-rich polymer linked to the core chain of *P. aeruginosa*. With this model, a loss of O side chain (smooth to rough form) would result in an apparent relative increase of rhamnose.

Adaptation to PB resistance in *P. aeruginosa* is a multifaceted and complicated phenomenon resulting in profound chemical and structural perturbations. It is probable, as suggested by Said et al. (26), that not all cellular alterations have a direct bearing on resistance. The correlation of specific alterations and resistance is unclear, and the molecular basis for adaptive resistance remains mechanistically unresolved. It is unlikely that the development of adaptive resistance to PB is the sole responsibility of a single component of the cell envelope such as LPS.

It is likely that alterations in LPS fatty acids are contributing factors but not solely responsible for adaptive resistance to PB in *P. aeruginosa*. Our results suggest a role for the 3-acyloxyacyl residues of LPS in the binding and biological activity of PB.

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